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Reconstitution of Natural Killer Cell Receptor Repertoires after Unmanipulated HLA-Mismatched/Haploidentical Blood and Marrow Transplantation: Analyses of CD94:NKG2A and Killer Immunoglobulin-Like Receptor Expression and Their Associations with Clinical Outcome

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ABSTRACT

The effect of natural killer (NK) cell alloreactivity on the outcome of haploidentical hematopoietic stem cell transplantation (HSCT), with or without in vitro T cell depletion, remains controversial. Killer immunoglobulin-like receptors (KIRs) recognize human leukocyte antigen C and B epitopes on target cells, thereby regulating NK cell activity. To examine the recovery of CD94:NKG2A and KIR (CD158a, CD158b, and CD158e) expression by NK cells, we used flow cytometry to evaluate samples from 24 patients and their donors before and in the year following unmanipulated HLA-haploidentical/mismatched blood and marrow transplantation. Linear regression analysis demonstrated that NKG2A recovery was inversely correlated with CD158b recovery in the year following transplant. The doses of T cell subgroups $CD4^+$ and $CD8^+$ were inversely associated with CD158a and CD158e expression during the 2 months following transplantation. Moreover, patients with grades II-IV acute graft-versus-host disease (aGVHD) or who received "high" doses of T cells (>1.37 × 10^8/kg) showed delayed recovery of KIRs during the 2 months following transplantation. Univariate analysis showed that patients with high CD94 expression by day 60 (>90%) or who received donors with high CD94 expression (>80%) were associated with higher transplantation-related mortality (P = .006 or .067, respectively) and poorer leukemia-free survival (P = .012 or .094, respectively). Thus, the occurrence of aGVHD or the receipt of high doses of T cells in the allograft altered KIR reconstitution. Furthermore, high levels of CD94 expression in donors or in recipients by day 60 might be a good predictor for poor prognosis.

KEY WORDS

HLA-mismatched • Haploidentical • HSCT • GVHD • KIRs • CD94:NKG2A

INTRODUCTION

Natural killer (NK) cells can discriminate "self" from "nonself" through their inhibitory natural killer receptor (NKR) expression. There are 2 types of inhibitory NKR. One type is the killer immunoglobulin-like receptor family (KIR), which includes inhibitory receptors for the polymorphic determinants of HLA-A (KIR3DL2), HLA-B (KIR3DL1), and HLA-C (KIR2DL1, KIR2DL2, and KIR2DL3). In addition to KIRs, the NKG2A lectin-like receptor has specificity for complexes of HLA-E and peptides derived from the leader sequences of many, but not all, HLA-A, HLA-B, and HLA-C polypeptides [1].

The effect of KIR ligand incompatibility in the graft-versus-host direction, which is associated with NK cell alloreactivity, on outcomes of haploidentical or mismatched unrelated hematopoietic stem cell

transplantation (HSCT) remains controversial [2-10]. The observed inconsistent roles of KIR ligand mismatch seem to arise from different transplant protocols with various extents of T cell depletion (TCD) in vitro or in vivo.

By evaluating the KIR expression of donor-derived NK cells by day 100 after unmanipulated bone marrow or TCD transplants, Cooley et al. [11] have demonstrated that KIR reconstitution is altered by T cells in the allograft and is associated with clinical outcome, which may partially account for the different roles of alloreactive NK cells in transplants. Shilling's previous report [12] showed that patients with idiosyncratic low KIR recovery on NK cells suffered from graft versus host disease (GVHD), early relapse, and other complications after HLA-matched HSCT. Nguyen et al. [13] reported that the impaired cytotoxicity of low KIR- and high NKG2A-expressing NK cells was associated with both patient death and relapse in TCD, haploidentical, KIR ligand-mismatched HSCT. Thus far, no report has evaluated the reconstitution kinetics of KIRs or their associations with clinical outcomes after HLA-mismatched/haploidentical HSCT without in vitro TCD. Recently, we established a novel protocol that includes conditioning with antithymocyte globulin (ATG) prior to unmanipulated HLA-mismatched/haploidentical blood and marrow transplantation. This protocol can achieve comparable outcomes to HLA-identical sibling transplantation [14,15]. Patients undergoing this protocol provide a unique model for analyzing KIR reconstitution in vivo under conditions of HLA-mismatch and high doses of T cells.

Therefore, the goal of this study was to examine KIR recovery on NK cells after HLA-mismatched/haploidentical HSCT (with T cell repletion). Specifically, we wished to assess any differences in KIR recovery that may affect the cytotoxicity and alloreactivity of NK cells and to compare results with those from studies of HLA-matched HSCT or HLA-mismatched HSCT (with TCD), as reported by Shilling et al. [12] and Nguyen et al. [13], respectively.

METHODS

Patients

Patients with hematologic malignancies suitable for allo-HSCT who lacked HLA-identical related or unrelated donors were candidates for the HLA-mismatched/haploidentical HSCT. Twenty-four patients with malignant hematologic disease who underwent haploidentical allo-HSCT between April 2004 and December 2004 were included in this study. All patients and their donors provided informed consent, and this study was approved by the institutional review board of Peking University Institute of Hematology. Patient characteristics including age, sex, underlying hematologic disorders, KIR ligand compatibility, the occurrence of aGVHD, and clinical outcomes are presented in Tables 1 and 2. Pretransplantation risk categories were low or high risk. High-risk diseases included acute myelogenous leukemia (AML) and acute lymphoblastic leukemia (ALL) other than first complete remission (CR1); chronic myelogenous leukemia not in the first chronic phase (CML not CP1); non-Hodgkin's lymphoma (NHL). Low-risk diseases were ALL CR1, AML CR1, and CML CP1.

Conditioning Regimen, Mobilization, Collection of Stem Cells, and Graft-versus-Host Disease (GVHD) Prophylaxis

All patients received myeloablative regimens. The drugs administered before transplantation in the regimens described below were cytosine arabinoside (Ara-C, 4 g/m^2 on days −10 and −9), busulfan (Bu, 12 mg/kg administered orally in 12 doses over 3 days, on days −8, −7, and −6), cyclophosphamide (Cy, 1.8 g/m^2 on days −5 and −4), simustine (Me-CCNU, 250 mg/kg on day −3), and ATG (10 mg/kg i.v., rabbit, on days −5 through −2; Sangstat) [14,15].

Donors received rhG-CSF (Filgrastim, 5 μg/kg daily for 5-6 days). On the fourth day, rhG-CSF-primed bone marrow cells (G-BM) were harvested. The target total nucleated cell count was 3.0×10^8 (median 3.6×10^8, range: $0.24\text{-}8.16 \times 10^8$) cells/kg recipient weight. On the fifth and sixth days, rhG-CSF-mobilized peripheral blood progenitor cells (G-PBPCs) were collected. The target mononuclear cell count was 3.0×10^8 (median 3.65×10^8, range: $2.77\text{-}11.7 \times 10^8$) cells/kg recipient weight. The fresh and unmanipulated G-BM and G-PBPCs were infused into the recipients on the day of collection [14,15].

Prophylaxis for GVHD included cyclosporine A (CsA) and short-term methotrexate (MTX) with mycophenolate mofetil (MMF) [14,15]. CsA was started i.v. on day −9 at the dosage of 2.5 mg/kg. As soon as the patient was able to take medication after engraftment, CsA was switched to the oral formulation. Whole blood CsA concentration was monitored weekly using a fluorescence polarization immunoassay, and the dosage was adjusted to achieve a blood concentration of 150-250 ng/mL. MMF was administered orally (0.5 g every 12 h) from day 9 before transplantation to day 30 after transplantation, then 0.25 g MMF was given every 12 h for 1-2 months. MTX (15 mg/m^2) was administered i.v. on day 1, and 10 mg/m^2 MTX was given on days 3, 6, and 11 after transplantation. The diagnosis and grading of GVHD was established according to published criteria [16].

Filgrastim (G-CSF, 5 μg/kg per day) was given subcutaneously to all recipients from day 6 after transplantation until the neutrophil count reached 0.5×10^9

Table 1. *Patient Characteristics*

No.	Age (y)	Sex	Diagnosis	Donor	Donor-Patient KIR Ligand#			aGVHD	Outcome (Days*)
					Recipient	Donor	Mismatch		
1	49	F	CML	Son	HLA-C1,BW4	HLA-C1	HVG	(−)	alive and CCR (770)
2	14	F	CML	Mother	HLA-C1	HLA-C1	(−)	II	Relapse (370)
3	33	M	CML	Sibling	HLA-C1	HLA-C1	(−)	(−)	Dead, TRM (290)
4	41	M	AML-M5	Sibling	HLA-C2,BW4	HLA-C1,C2,BW4	GVH	IV	Dead, TRM (90)
5	22	M	ALL	Mother	HLA-C1,C2,BW4	HLA-C1,C2,BW4	(−)	II	alive and CCR (740)
6	16	M	CML	Mother	HLA-C1,C2,BW4	HLA-C1,C2	HVG	(−)	alive and CCR (733)
7	49	M	AML-M4a	Sibling	HLA-C1,BW4	HLA-C1	HVG	III	alive and CCR (724)
8	7	M	ALL	Father	HLA-C1	HLA-C1,C2	GVH	II	Relapse (330)
9	16	F	ALL	Mother	HLA-C1,BW4	HLA-C1,BW4	(−)	(−)	alive and CCR (700)
10	8	M	NHL-IIIB	Mother	HLA-C1	HLA-C1	(−)	(−)	Dead, TRM (60)
11	40	F	CML	Sibling	HLA-C1,C2	HLA-C1,C2	(−)	(−)	alive and CCR (680)
12	13	M	AML-M2	Mother	HLA-C1,BW4	HLA-C1,BW4	(−)	IV	alive and CCR (567)
13	19	F	NHL-IVA	Mother	HLA-C1,BW4	HLA-C1,BW4	(−)	(−)	Dead, TRM (270)
14	50	M	AML-M5	Sibling	HLA-C1,BW4	HLA-C1,BW4	(−)	(−)	Dead, TRM (200)
15	30	F	CML	Mother	HLA-C1,BW4	HLA-C1,BW4	(−)	II	alive and CCR (605)
16	41	M	CML	Sibling	HLA-C1,C2,BW4	HLA-C1,C2,BW4	(−)	(−)	alive and CCR (594)
17	28	F	CML	Sibling	HLA-C1,BW4	HLA-C1	HVG	II	Dead, TRM (355)
18	10	F	CML	Mother	HLA-C1,BW4	HLA-C1,BW4	(−)	II	alive and CCR (560)
19	10	M	AML-M2	Father	HLA-C1	HLA-C1,BW4	GVH	II	alive and CCR (546)
20	16	M	ALL	Mother	HLA-C2,BW4	HLA-C2,BW4	(−)	(−)	alive and CCR (531)
21	39	F	CML	Sibling	HLA-C1,C2,BW4	HLA-C1,C2,BW4	(−)	II	alive and CCR (495)
22	30	M	CML	Sibling	HLA-C1	HLA-C1	(−)	II	alive and CCR (520)
23	23	F	CML	Mother	HLA-C1,C2	HLA-C1	HVG	II	alive and CCR (509)
24	18	M	AML	Mother	HLA-C1	HLA-C1	(−)	(−)	alive and CCR (500)

F indicates female; M, male;

#GVH, donor-patients KIR ligand GVH direction mismatch; HVG, donor-patients KIR ligand HVG direction mismatch;

CCR continuous complete remission; CML, chronic myelogenous leukemia; AML, acute myelogenous leukemia; NHL, non-Hodgkin's lymphoma; aGVHD, acute graft-versus-host disease; HVG, host versus graft; TRM, transplant-related mortality.

*Days after HSCT at last follow-up.

cells/L for 3 consecutive days [14,15]. Bone marrow aspiration and cytogenesis studies to assess engraftment were performed 1, 2, and 3 months after transplantation. HLA DNA typing and PCR-DNA fingerprinting (short tandem repeat) were used for donor chimerism detection. For each patient, at least 2 methods were used to confirm donor chimerism [14,15].

Sample Collection

Peripheral blood (10 mL) samples were drawn from donors and from patients before transplantation and at days 30, 60, 90,120, and 180 and at 1 year after transplantation. All samples were drawn within 4 days of the specified days. Samples were available for 24 patients and their paired donors before transplantation, for 24 patients at days 30 and 60, for 20 patients at day 120, for 16 patients at day 180, and for 12 patients at 1 year.

Antibodies and Flow Cytometry Analysis

Table 3 shows the combinations of monoclonal antibodies (mAbs) used in individual 4-color or 3-color flow cytometry assays to analyze KIR and CD94:NKG2A expression on NK cells or to analyze the immunophenotype recovery of $CD56^{+}$ NK cells and the NK cell subsets $CD56^{dim}$ and $CD56^{bright}$. A minimum of 100,000 nucleated, cell-gated events were acquired for each specimen. After gating on the CD3-$CD56^{+}$ lymphocyte (ie, NK cell), the KIR- or CD94:NKG2A-expressing populations were identified in the presence of an irrelevant isotype-matched control antibody to set the lower limit of the KIR- or CD94:NKG2A-positive gate, thereby excluding background fluorescence and receptor-negative cells. Acquisition and analyses were performed with CellQuest software (Beckton-Dickinson, San Jose, CA).

KIR Ligand Typing and Grouping

HLA-A and -B were typed with serologic methods. HLA-C and -DRB1 alleles were typed with PCR-SSP (sequence-specific primer) as per the manufacturer's instructions (Pel-Freez Biological, Rogar, AR). The HLA-C cells were classified into 2 groups: group 1 (HLA-C1; including the HLA-Cw1, 3, 7, 8, 13, and 14 alleles) and group 2 (HLA-C2; including the HLA-Cw2, 4, 5, 6, 12, 15, 17, and 18 alleles) [17]. CD158a (KIR2DL1/2DS1) was specific for HLA-C1, CD158b (KIR2DL2/2DL3/2DS2) was specific for HLA-C2, and CD158e (KIR3DL1) was specific for the HLA-B allotypes expressing the serologic Bw4 epitope ($HLA\text{-}B^{Bw4}$) [9].

Table 2. *Patient Characteristics of the "High" and "Low" CD94 Expression Groups*

	Group A	Group B	*P* Value	Group C	Group D	*P* Value
N	12	12		12	12	
Median patient age, years (range)	23 (8-50)	28 (7-54)	NS	28 (8-50)	20 (7-54)	NS
Median donor age, years (range)	42 (26-58)	39 (20-50)	NS	41 (26-58)	41 (35-43)	NS
Patent sex, male, n (%)	6 (50%)	8 (67%)	NS	7 (58%)	7 (58%)	NS
Diagnosis			0.044			0.044
AML, n (%)	5 (42%)	1 (8%)		5 (42%)	1 (8%)	
ALL, n (%)	1 (8%)	3 (25%)		1 (8%)	3 (25%)	
CML, n (%)	4 (33%)	8 (67%)		4 (33%)	8 (67%)	
NHL, n (%)	2 (17%)	0 (0%)		2 (17%)	0 (0%)	
Pretransplantation risk categories*			NS			NS
High risk, n (%)	5 (51.7%)	4 (33.3%)		6 (50%)	3 (25%)	
Numbers of Patient/donor HLA incompatibility (HLA-A, -B, -DR)			NS			NS
1 locus, n (%)	2 (17%)	3 (25%)		2 (17%)	2 (17%)	
2 loci, n (%)	6 (50%)	5 (42%)		7 (58%)	5 (42%)	
3 loci, n (%)	4 (33%)	4 (33%)		3 (25%)	5 (42%)	
Patient/donor KIR ligand			NS			NS
Match, n (%)	9 (75%)	7 (58%)		10 (83%)	6 (50%)	
HVG mismatch, n (%)	2 (17%)	3 (25%)		1 (8%)	4 (33%)	
GVH mismatch, n (%)	1 (8%)	2 (17%)		1 (8%)	2 (17%)	
II-IV aGVHD, n (%)	7 (58%)	6 (50%)	NS	7 (58%)	6 (50%)	NS
cGVHD, n (%)	5/10 (50%)	7/12 (58%)	NS	6/10 (60%)	6/12 (50%)	NS
Median (range)						
CD34 infused,$\times 10^6$/kg	2.39 (1.07-5.97)	2.38 (1.45-5.58)	NS	2.07 (1.07-5.97)	3.0 (1.45-5.58)	NS
CD3 infused,$\times 10^8$/kg	1.90 (0.44-5.79)	1.5 (1.00-2.87)	NS	1.48 (0.44-3.19)	1.79 (1.18-5.79)	NS

The median expression of CD94 on NK cells in donors was 80% and that in patients by day 60 were 90%. Patients were classified into group A and group B according to greater than or lower than the median CD94 expression of their donors, or were divided into group C and group D based on greater than or lower than the median CD94 expression of patients by day 60.

AML indicates acute myelogenous leukemia; CML, chronic myelogenous leukemia; ALL, acute lymphoblastic leukemia; CR, complete remission; CP, chronic phase; NHL, non-Hodgkin's lymphoma; aGVHD, acute graft-versus-host disease; KIR, killer immunoglobulin-like receptors.

*Pretransplantation risk categories were low or high risk. High-risk diseases included AML and ALL other than CR1; CML not CP1; NHL. Low-risk diseases were ALL CR1, AML CR1, and CML CP1.

Statistical Analysis

Data as of June 1, 2006, were analyzed. The calculations were carried out with SPSS statistical software. The nonparametric Wilcoxon test was used to compare the distributions of KIR and CD94:NKG2A expression levels. To test the differences in levels between donors and recipients paired after transplantation, a Wilcoxon signed rank test was used. A 2-sided Mann-Whitney *U* test was applied to compare differences in KIR expression between the 2 categories of patients. Leukemia-free survival (LFS) was estimated using Kaplan-Meier methods. The probabilities of relapse, transplant-related mortality (TRM), and grades II-IV acute GVHD (aGVHD) were calculated as cumulative incidences to adjust the analysis for competing risks. Log-rank test statistics were used to evaluate the univariate effects of "high" CD94 expression on outcome. Linear regression analysis was performed to examine the relationship between 2 continuous measures of the expression of 2 proteins, or between the infused dose of T cell subgroups and the early recovery of KIRs. $P < .05$ was considered statistically significant.

RESULTS

Clinical Outcomes

Engraftment and complete donor chimerism after transplantation were achieved by all patients. As of

Table 3. *Combinations of Fluorochrome-Labeled Monoclonal Antibodies (mAb) Used for Immunophenotyping*

	FITC-Ab	PE-Ab	PerCP-Ab	APC-Ab
CD158a/CD158b	CD158a*	CD158b*	CD3	CD56
CD158e	CD158e*		CD3	CD56
CD94:NKG2A	CD94*	NKG2A†	CD3	CD56
NK subset	CD16	CD56	CD45	CD3

*From PharMingen (San Diego, CA).

†From Beckman-Coulter-Immunotech (Brea, CA). The others were from Becton-Dickinson (San Jose, CA).

June 1, 2006, 16 of the 24 patients survived without leukemia, 6 patients died of TRM, and 2 patients relapsed on day 370 and 330 after HSCT, respectively.

Reconstitution Kinetics of CD94:NKG2A and KIR (CD158a, CD158b, CD158e) Expression by NK Cells after Transplantation

The quantitative evaluation of CD94:NKG2A and KIR expression on the surface of circulating NK cells after unmanipulated HLA-mismatched/haploidentical blood and marrow transplantation was performed by flow cytometry in a series of 24 patients and their paired donors. Because the occurrence of aGVHD and its therapy may affect immune recovery, only the 7 patients who survived without leukemia and were exempt from grades II-IV aGVHD were considered suitable for evaluation of the reconstitution kinetics of CD94:NKG2A and KIRs on NK cells.

As shown in Figure 1A and B, the expression of CD94 and NKG2A on NK cells in recipients became significantly higher than donor levels on day 30 after HSCT (P = .043 and .018, respectively), then sequentially decreased from day 60 to 1 year. The levels of CD94 expression on NK cells by day 60 after transplantation were not significantly different than donor levels (P = .128; Figure 1A). In contrast, the levels of NKG2A expression on NK cells at 1 year were still higher than donor values (P = .043; Figure 1B). Although the expression of all KIRs (CD158a, CD158b, and CD158e) after transplantation was equal to the donor levels ($P >$ .1), the reconstitution kinetics of CD158a and CD158b expression were different from those of CD158e. The expression of CD158a and CD158b diminished by day 30 after HSCT in patients (P = .398 and .398, respectively), and then sequentially increased from day 60 to 1 year after HSCT ($P >$ .1; Figure 1C and D). However, the reconstitution kinetics of CD158e expression on NK cells resembled those of CD94 or NKG2A expression, increasing first from day 30 to 60 (P = .310 and .499), and then sequentially decreasing from day 120 to 1 year after HSCT ($P >$ .1; Figure 1E).

NKG2A Expression Was Inversely Correlated with CD158b Expression after Transplantation

At day 30, a significant inverse correlation between the expression of NKG2A and total HLA-C-binding KIR (CD158a and CD158b) expression, in particular CD158b expression, was found only in patients with grades 0-I aGVHD (n = 11; Figure 2A). However, at the rest individual time points after transplantation (days 60, 120, 180, and 1 year, respectively; Figure 2B-E), the expression of NKG2A was inversely correlated with that of CD158b in all observed patients (n = 24, 20, 16, and 12, respectively). No correlation between NKG2A and CD158a or CD158e expression was found before or after transplantation.

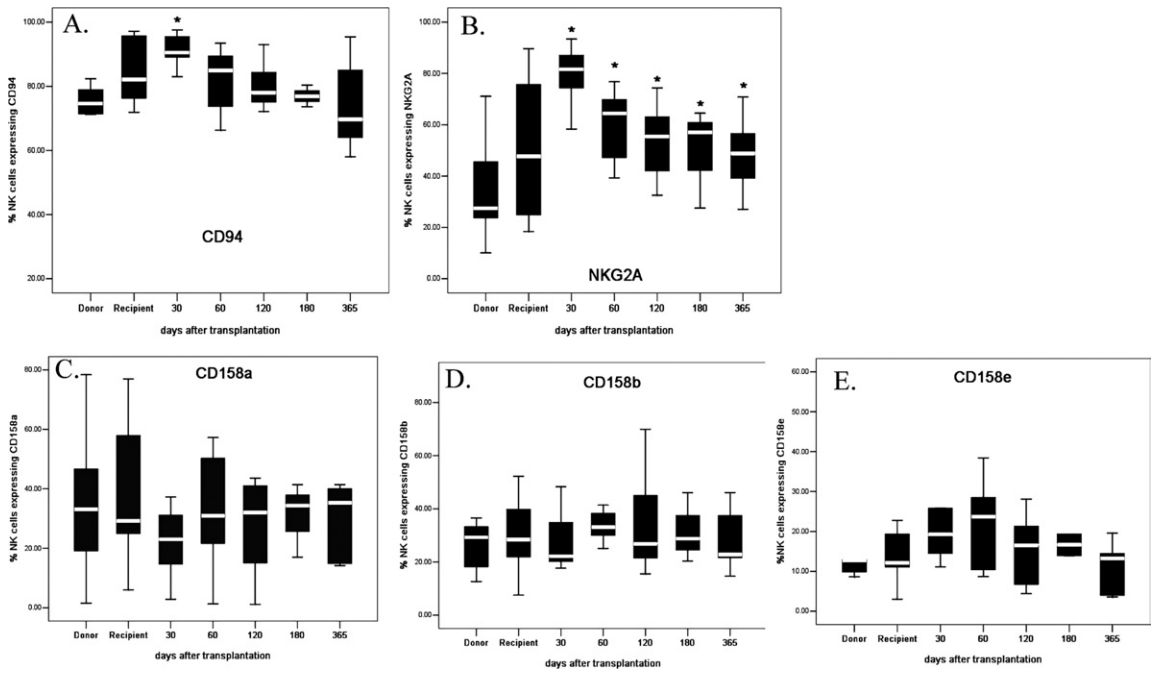

Figure 1. Reconstitution kinetics of CD94:NKG2A and KIR expression by NK cells after transplantation. The median, 25% to 75% percentile ranges, and extreme values of the expression of CD94 (A), NKG2A (B), and KIRs on NK cells (C-E) were shown in the box plot (n = 7). The asterisks (*) represent $P <$ .05 compared with the expression levels of the donors.

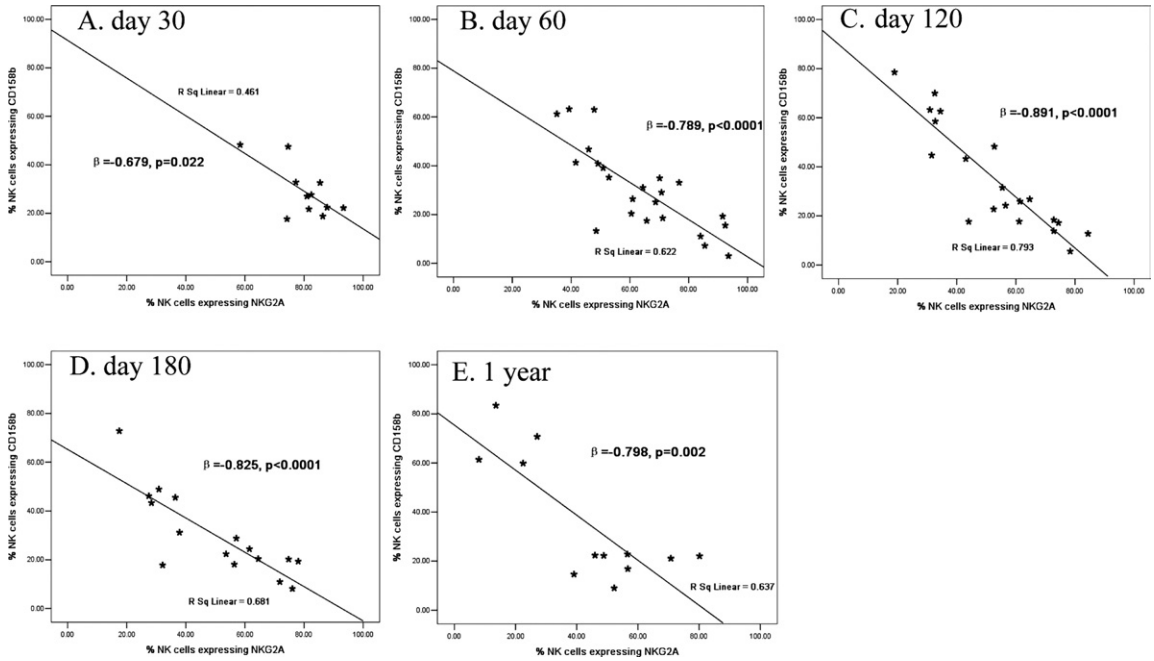

Figure 2. NKG2A expression was inversely correlated with CD158b expression after transplantation. The inverse correlation between NKG2A expression and CD158b expression were shown in patients without II-IV aGVHD by day 30 (n = 11) (A), by day 60 (n = 24) (B), by day 120 (n = 20) (C), by day 180 (n = 16) (D), and by 1 year (n = 12) after transplantation (E).

Acute GVHD Altered KIR Reconstitution after Transplantation

Among the 24 patients in the present study, 11 developed grades 0-I aGVHD and 13 developed grades II-IV aGVHD. CD94:NKG2A reconstitution was little influenced by the occurrence of grades II-IV aGVHD or its therapy. KIR expression on NK cells had reached donor levels within 1 month in patients who were exempt from grades II-IV aGVHD, or within 4 months in patients with grades II-IV aGVHD (Figure 3). In patients of the grades II-IV aGVHD (n = 13), expression on NK cells of CD158a by day 30 and 60 (P = .012 and .050; Figure 3A) and of CD158b by day 30 (P = .005; Figure 3B) was significantly lower than donor levels, but expression on NK cells of CD158e (Figure 3C) was similar to that of the donors. Furthermore, CD158b expression on NK cells in patients with grades II-IV aGVHD was lower than in patients with grades 0-I aGVHD by day 30 (P = .018). KIR expression on NK cells in donors of patients with grades II-IV aGVHD was similar to that in donors of patients with grades 0-I aGVHD (n = 11, data not shown).

Doses of T Cell Subgroups Infused Were Inversely Correlated with the Levels of CD158a or CD158e Expression after Transplantation

Linear regression analysis demonstrated a significant inverse correlation between the dose of $CD4^+$ T cells infused and CD158a expression at days 30 and 60 ($\beta = -0.517$, P = .014, n = 24; Figure 4A; $\beta = -0.492$, P = .024, n = 24; Figure 4B; respectively). A significant inverse correlation between dose of $CD8^+$ T cells infused and CD158e expression at days 30 and

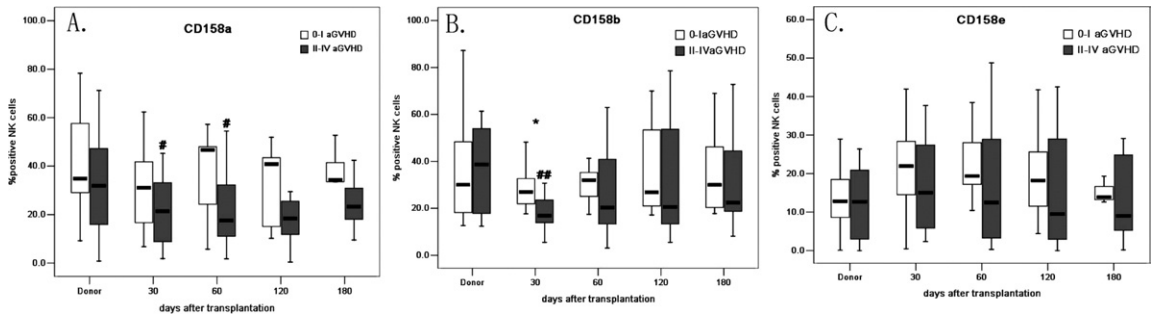

Figure 3. Acute GVHD altered KIR reconstitution after transplantation. Effect of grades 0 -I (open column) versus II-IV (closed column) aGVHD on CD158a (A), CD158b (B), and CD158e reconstitution (C). *P < .05 between patients with grades 0-I (n = 11) and II-IV aGVHD (n = 13), ##P < .01 or #P < .05 compared with the expression levels of the donors.

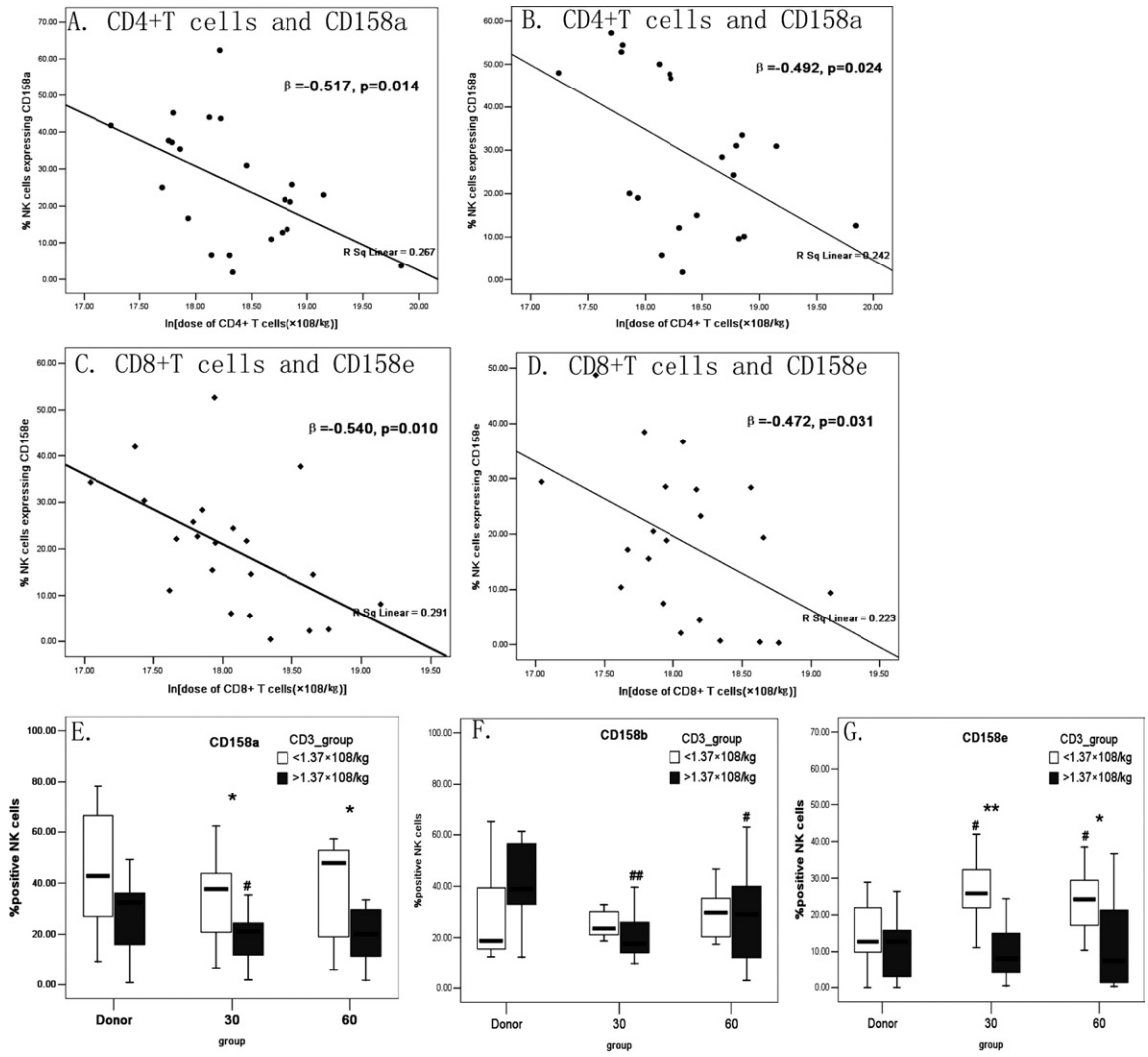

Figure 4. Doses of T cell subgroups infused were inversely correlated with the levels of CD158a or CD158e expression after transplantation. Inverse correlation between the dose of $CD4^+$ T cells infused and CD158a expression at days 30 (n = 24) and 60 (n = 24) (A, B), or between the dose of $CD8^+$ T cells infused and CD158e expression at days 30 (n = 24) and 60 (n = 24) (C, D). Effect of "high" (filled column, n = 12) versus "low" (open column, n = 12) dose of T cells on CD158a (E), CD158b (F), and CD158e recovery (G). $^{**}P < .01$or $^{*}P < .05$ between patients of the "high" and "low" T cell groups; $\#\#P < .01$or $\#P < .05$ compared with the expression levels of the donors.

60 ($\beta = -0.540$, $P = .010$, n = 24; Figure 4C; $\beta = -0.472$, $P = .031$, n = 24; Figure 4D; respectively) was also revealed. No correlation was found between doses of T cell subgroups infused and CD158b expression in patients after transplantation.

The median dose of $CD3^+$ cells in allograft of the 24 patients was 1.37×10^8/kg. Patients were classified into "low" or "high" T cell groups based on whether they received less or more than the median $CD3^+$ cell dose. In patients of the "high" T cell group (n = 12), expression on NK cells of CD158a ($P = .021$ and .075; Figure 4E) and of CD158b ($P = .004$ and .026; Figure 4F) by day 30 and 60 was significantly lower than donor levels, but CD158e expression in the "high" group patients was similar to that of the donors (Figure 4G). In contrast, in patients of the "low" T cell group (n = 12), CD158a and CD158b expression on NK cells was similar to the levels of the donors, whereas CD158e expression was significantly increased compared with the expression levels of the donors by day 30 and 60 ($P = .033$ and .037; Figure 4G). Furthermore, in patients of the "high" T cell group, expression on NK cells of CD158a ($P = .028$ and .029; Figure 4E) and of CD158e ($P = .003$ and .024; Figure 4G) was lower than in patients of the "low" T cell group by days 30 and 60, respectively. KIR expression on NK cells in donors of patients in the "high" T cell group was similar to that in donors of patients in the "low" T cell group (data not shown).

Increased CD94 Expression Correlated with Poor Survival

Up to June 1, 2006, 16 patients were alive who were all free of their original leukemia through follow-up with

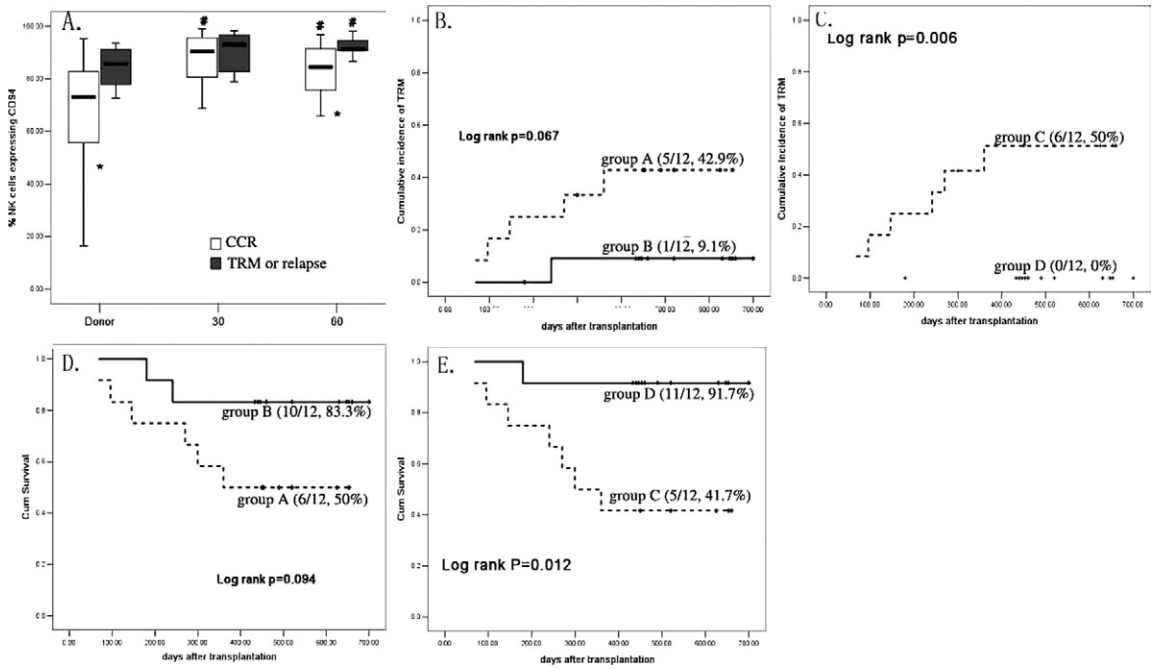

Figure 5. Increased CD94 expression correlated with poor survival. Different levels of CD94 expression on NK cells between patients that died of TRM/relapse (A) (filled column, n = 8) versus those patients alive with CCR (open column, n = 16), or between their respective donor groups. Cumulative incidence estimates of TRM for patients of group A (n = 12) (B) and group B (n = 12), or for patients of group C (n=12) (C) and group D (n = 12); Kaplan-Meier survival estimates LFS for patients of group A and group B (D), or for patients of group C and group D (E). *$P < .05$ between the black and white column group. #$P < .05$ compared with the expression levels of the donors.

a median of 580 days (range: 495-770 days). Data as of June 1, 2006, were analyzed. As shown in Figure 5A, the levels of CD94 expression on NK cells were significantly higher in patients after transplantation compared with their donors. The levels of CD94 expression in donors of patients with TRM or relapse (n = 8) were significantly higher than in donors of patients who were alive with continuous complete remission (CCR, n = 16) ($P = .029$; Figure 5A). By day 60, the levels of CD94 expression were significantly higher in patients who subsequently died of TRM or relapse (n = 8) than in patients alive with CCR (n = 16) ($P = .027$; Figure 5A). The median expression of CD94 on NK cells in donors was 80%, and that in patients by day 60 were 90%. To evaluate the predictive role of CD94 expression for LFS, patients were classified into group A (n = 12) and group B (n = 12) according to greater than or lower than the median CD94 expression of their donors, or were divided into group C (n = 12) and group D (n = 12) based on greater than or lower than the median CD94 expression of patients by day 60. "High" levels of CD94 expression in donors (group A) or in patients by day 60 (group C) were associated with a higher cumulative incidence of TRM (42.9% versus 9.1%, $P = .067$; Figure 5B; 50% versus 0%, $P = .006$; Figure 5C) and a lower cumulative incidence of 2-year LFS (50% versus 83.3%, $P = .094$; Figure 5D; 41.7% versus 91.7%, $P = .012$; Figure 5E) compared with "low" levels of CD94 expression in donors (group B) or in patients by day 60 (group D), respectively. There were no differences in the cumulative incidence of relapse between group A and group B (12.5% versus 8.3%, $P = .856$) or between group C and group D (14.3% versus 8.3%, $P = .793$). As shown in Table 2, no substantial differences were found between group A and group B or between group C and group D except for the disease diagnosis ($P = .044$).

Lymphocyte Reconstitution after Transplantation

To assess whether KIR reconstitution patterns reflected underlying differences in hematopoietic recovery, the rates of lymphocyte reconstitution in white blood cells (WBC) were analyzed by evaluating the count and proportions of total lymphocytes, total T cells, and total NK cells (with NK subpopulations $CD56^{dim}$ and $CD56^{bight}$). There were no significant differences in the reconstitution rates between patients receiving "high" (n = 12) versus "low" doses of T cells (n = 12) or between patients of group A (n = 12) and group B (n = 12), or between patients of group C (n = 12) and group D (n = 12) after transplantation (data not shown). However, a significantly delayed recovery of the total NK cell proportion in WBC was observed in patients with grades II-IV aGVHD (n = 13) compared to patients with grades 0-I aGVHD (n = 11) by day 30 after transplantation

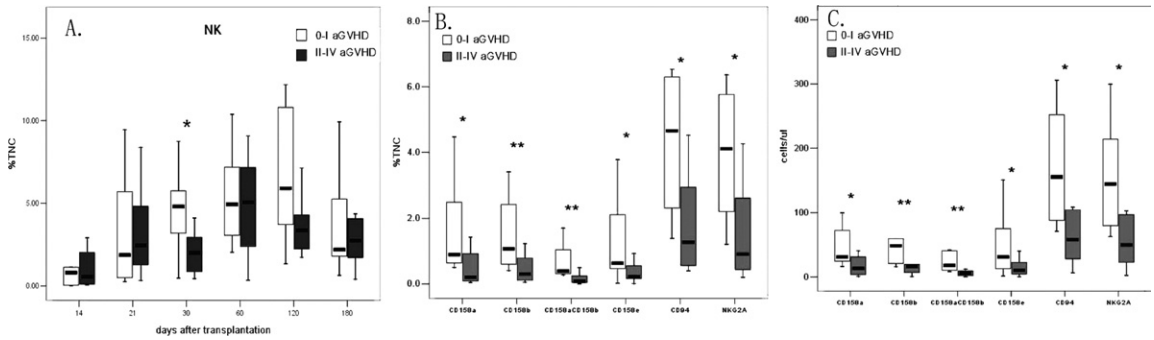

Figure 6. Acute GVHD altered NK reconstitution after transplantation. Effect of grades 0 -I (open column) versus II-IV (closed column) aGVHD on the absolute proportion in WBC (TNC%) of overall NK cells (A), of KIR positive or CD94 (B): NKG2A positive NK cells; and on the absolute number in WBC per microliter (Cells/μl) of KIR positive or CD94:NKG2A positive NK cells (C). *$P < .05$ or **$P < .01$ between patients with grades 0-I (n = 11) and II-IV aGVHD (n = 13).

($P = .030$; Figure 6A). Accordingly, both the absolute proportions and counts of KIR-, CD94-, or NKG2A-positive NK cells in WBC were significantly reduced in patients with grades II-IV aGVHD ($P < .05$; Figure 6B and C). There were no significant differences in the ratio of $CD56^{dim}$ to $CD56^{bright}$ cells from patients with grades II-IV aGVHD versus grades 0-I aGVHD ($P = .893$).

DISCUSSION

In agreement with previous studies [11-13,18], we found that CD94:NKG2A was the predominant NK cell receptor in the early posttransplantation period, but CD94:NKG2A expression decreased as KIR expression by NK cells increased. The expression of NKG2A and HLA-C-binding KIRs (especially CD158b) was inversely correlated during reconstitution, suggesting that these 2 types of receptors share a common purpose and some degree of coordination in their differential expression during NK cell development. These data provide evidence that the potential for NKR repertoire recovery is a genetically programmed process [19] after HLA-mismatched/haploidentical HSCT (with T cell repletion), which is similar to what was observed for HLA-matched transplant [12] and HLA-mismatched transplant with TCD [13]. The different recovery kinetics of HLA-C-binding KIRs (CD158a and CD158b) versus an HLA-Bw4-binding KIR (CD158e) were also confirmed [11], but the clinical significance should be explored in the future.

The time for KIR reconstitution after different transplant protocols varies from 5 to 36 months, as described by Shilling et al. [12] and Nguyen et al. [13]. In our transplantation setting, we observed that KIR expression on NK cells had already reached donor levels within 1 month in patients who survived without leukemia and who were exempt from grades II-IV aGVHD, and within 4 month in patients with grades II-IV aGVHD, although T cells in the allograft might delay KIR reconstitution as reported by Cooley et al. [11]. Therefore, the reconstitution kinetics of KIRs after using the present protocol could be quicker than those after HLA-mismatched transplant with TCD or HLA-matched related or unrelated transplant [12,13]. Several possible factors, including genetic incompatibility between donor and recipient as well as TCD or the use of intensive conditioning with total body irradiation (TBI), may be responsible for different rates of KIR reconstitution [12,13,18,19]. Although not yet determined, we anticipate that the use of a mixture of G-BM and G-PBPCs in the graft might be a factor in the accelerated KIR reconstitution observed in the present transplant protocol, in addition to the high degree of HLA-mismatch.

The effects of aGVHD and T cells on the recovery of KIRs were also explored in our transplantation protocol. As far as aGVHD is concerned, because of the delayed recovery of CD158b in some patients by day 30, the inverse correlation between NKG2A and CD158b was only existed in the 11 patients without grades II-IV aGVHD. This finding suggests that aGVHD or its therapy has a predominantly inhibitory effect on the recovery of HLA-C group 2-binding KIRs (CD158b, KIR2DL2/2DL3/2DS2). Moreover, aGVHD and its therapy also delayed the recovery of the proportion of NK cells in WBC. The inverse correlation between T cell subpopulations and the early recovery of CD158a or CD158b provided direct evidence that T cells in the allograft may have a predominantly inhibitory effect on the recovery of HLA-C group 1-binding KIRs (CD158a, KIR2DL1/2DS1) and HLA-Bw4-binding KIRs (CD158e, KIR3DL1). However, the overall proportion and count of NK cells in WBC were less affected by "high" doses of T cells (data not shown). Thus, a preliminary conclusion could be drawn that "high" doses of T cells influence the quality rather than the

quantity of NK cells, whereas aGVHD and its therapy impair both NK cell quality and quantity.

The NK cell population can be further divided into $CD56^{bright}$ and $CD56^{dim}$ subsets. These phenotypes may reflect different stages in NK development or distinct regulatory and cytolytic NK subpopulations [20]. The ratio of $CD56^{dim}$ to $CD56^{bright}$ cells was similar in patients with grades II-IV aGVHD versus grades 0-I aGVHD and in patients with "high" doses of T cells versus "low" doses. These findings suggest that reduced KIR expression on NK cells in patients with grades II-IV aGVHD or in patients with "high" doses of T cells could not be attributed to an altered ratio of $CD56^{dim}$ to $CD56^{bright}$ cells.

Administration of ATG prior to transplantation results in donor TCD in vivo. However, in the context of similar dose of ATG (10 mg/kg) in the 24 patients, the in vivo TCD effects of ATG might be different between patients of the "high" and "low" T cell groups. In light of Cooley's data [11], T cells in the allograft might affect NK cell function and KIR expression in vivo after unrelated HSCT. In the present data, KIR reconstitutions were delayed in patients of the "high" T cell group instead of in patients of the "low" T cell group, suggesting that in vivo TCD effects of ATG were weakened in "high" T cell group. Our retrospective analysis has demonstrated that KIR ligand mismatch showed deleterious effects on clinical outcomes, especially in the "high" T cell group [7], which provides some evidence for this hypothesis.

We also showed for the first time that "high" CD94 expression in donors and in patients by day 60 were associated with higher TRM and poorer LFS. The characteristics of patients with "high" versus "low" CD94 expression were similar, except for the disease diagnosis ($P = .044$; Table 2). Moreover, the rates of LFS among patients with different diagnoses (AML, CML, ALL, or NHL) were similar (data not shown). Thus, the significantly different clinical outcomes cannot be explained by the different disease diagnoses between groups. Borrego et al. [21] demonstrated that the CD94 receptor can block CD69-initiated cytotoxic effects of NK cells. Therefore, "high" CD94 expression on NK cells might inhibit the cytotoxic effects of NK cells, which may cause higher TRM, resulting in poorer survival. On the whole, the characteristics of patients in group A were nearly the same as those in group C, and those in group B were almost equivalent to group D. Therefore, we propose that CD94 expression levels in donors may aid investigators in donor selection.

In summary, this is the first study to describe the reconstitution kinetics of the NKR repertoire after HLA-haploidentical/mismatched T cell-replete HSCT. Our results suggest that the occurrence of aGVHD or the receipt of a high dose of T cells in the allograft alters KIR reconstitution. Furthermore, high levels of CD94 expression in donors or in recipients by day 60 might be a good predictor for prognosis. Potential correlations between KIR reconstitution and other transplant-related complications, such as infection, are presently being investigated at our institute.

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